SOLUBILIZATION OF A FUNCTIONALLY ACTIVE PROLINE CARRIER FROM MEMBRANES OF *Escherichia coli* WITH AN ORGANIC SOLVENT

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SUMMARY

A proline transport carrier was extracted from the membranes of *Escherichia coli* with acidic *n*-butanol. Vesicles reconstituted from the butanol extract and *E.coli* phospholipids and preloaded with K^+ showed rapid uphill uptake of proline when energy was supplied as a membrane potential introduced by K^+-diffusion *via* valinomycin. Proline uptake by the reconstituted vesicles, like that of intact cells and isolated membrane vesicles, was inhibited by 3,4-dehydroproline, SH reagents, and a proton conducting uncoupler. Reconstituted vesicles of mutants defective in proline transport showed little or no proline uptake. The proline carrier was partially purified from the extract and separated from the bulk of phospholipids on Sephadex LH-20.

INTRODUCTION

Recently, biochemical studies on the mechanism of active transport in bacteria have progressed rapidly due to introduction of the technique of osmotic shock (1) and use of isolated membrane vesicles (2). The source of energy and the mechanism of energy coupling in uphill transport of solutes have been analyzed (3-6) and reviewed (7).

Genetic analyses of certain transport systems have shown that the transport carrier is essential for translocation of solutes across the membranes (8-10). These transport carriers may be defined as integral membrane proteins which bind solutes stereospecifically and translocate them across the membranes. No carrier protein has yet been isolated from *Escherichia coli* membranes, but protein fractions with carrier activities have recently been solubilized from other membranes using appropriate detergents (11-14). For instance, Kasahara and Hinkle (11) found that a protein fraction from the membranes of human erythrocytes carried out facilitated diffusion of D-glucose when reconstituted into liposomes. Hirata *et al.* (12) observed that the alanine carrier solubilized from the thermophilic

bacterium PS3 when reconstituted into vesicles with phospholipids took up substrate on supply of energy as a membrane potential introduced by K^+-diffusion *via* valinomycin.

This paper reports the solubilization of a proline carrier from the membranes of *E.coli*. This carrier, solubilized with an organic solvent, acidic *n*-butanol, was found to catalyze stereospecific uphill transport of proline when reconstituted into vesicles with *E.coli* phospholipids and supplied with energy in the form of a membrane potential.

MATERIALS AND METHODS

Materials --- Uniformly labeled L-[^{14}C]proline (290 mCi per mmole) was purchased from the Radiochemical Centre, Amersham, valinomycin from Calbiochem, and Sephadex LH-20 from Pharmacia. SF6847 (3,5-di-*tert*-butyl-4-hydroxybenzylidenemalononitrile) (15, 16) was kindly supplied by Dr. Y. Nishizawa, Sumitomo Chemicals, Osaka. Total phospholipids of *E.coli* were prepared from freshly harvested cells by the method of Bleigh and Dyer (17) and purified by chromatography on a silica gel column (Unisil, Clarkson Chemicals). Cholesterol was kindly given by Dr. K. Inoue, University of Tokyo.

Bacterial Strains and Growth Conditions --- *E.coli* strain W1-1 (Leu^-) was used unless otherwise stated. Cells, grown in a peptone-glycerol medium (18), were harvested in the late exponential phase of growth and stored at -70°. Strain JE2133 (*pro*A) (kindly given by Dr. M. Tomoeda, Kanazawa University), and its derivatives PT21 and PT22 were also used. The latter two, selected in this laboratory as strains requiring a high concentration of proline for growth, were found to be defective in proline transport (K. Motojima and Y. Anraku, manuscript in preparation). Cytoplasmic membrane vesicles (19) prepared from these mutants did not take up proline in the presence of D-lactate as a respiratory substrate. These strains were grown in CRM-glycerol medium (20) supplemented with 400 μg per ml of proline.

Preparation of the Membrane Fraction --- All procedures were carried out at 0-4°. Cells were washed once with 10 mM Tris-HCl (pH 7.3)-30 mM NaCl and resuspended in the same buffer containing 2.9 mM β-mercaptoethanol, 5 mM $MgCl_2$, and 2 μg per ml each of pancreatic DNase-I (Sigma) and RNase-A (Sigma). The cell density was adjusted to 1 g wet weight of cells per 3 ml of suspension. Then aliquots (40 ml) of the suspension were sonicated intermittedly for 10 min (Branson, model B-12, 80 W). Unbroken cells were removed by centrifugation at 4,500 x*g* for 10 min and the membrane fraction was precipitated by centrifugation at 114,000 x*g* for 30 min. The membranes were washed twice with 5 mM Tris-HCl (pH 7.3) containing 2.9 mM β-mercaptoethanol and 2.5 mM EDTA in a volume equal to that of the original cell-free supernatant, and used immediately.

Butanol Extraction of Membranes --- The method was similar to that used for extraction of C_{55}-isoprenoid alcohol phosphokinase from the membranes of *Staphylococcus aureus* (21). The washed membranes were evenly suspended in distilled water (40-50 mg protein per ml), and then 0.6 vol of *n*-butanol and 0.22 vol of glacial acetic acid (adjusted to pH 4.3 with pyridine) were added. The mixture was stirred for 40 min at 15-20° and then centrifuged for 15 min at 24,000 x*g* and 4°, and the dark brown butanol layer (butanol extract) was collected.

Preparation of Reconstituted, K^+-Loaded Vesicles --- The butanol extract containing 80-800 μg of protein was evaporated to dryness in a rotary evaporator at below 30° and kept *in vacuo* for at least 16 hr at 18-20°. Then 0.25 ml of *E.coli*

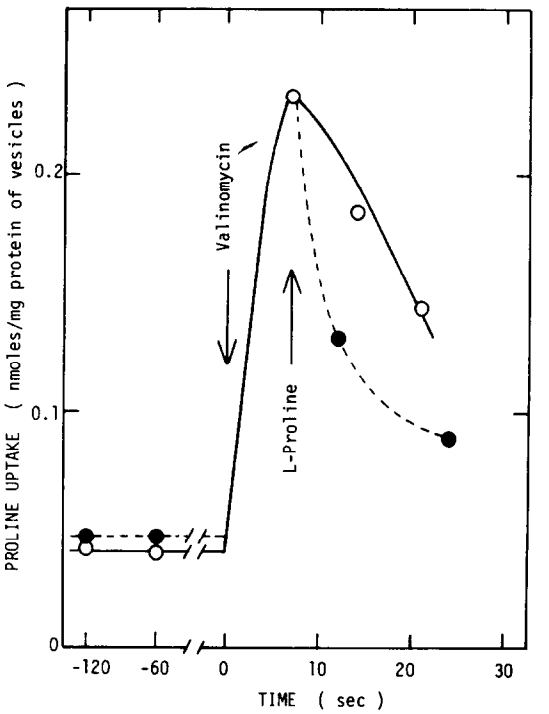

Figure 1. Proline uptake by reconstituted, K^+-loaded vesicles. Vesicles loaded with K^+ were reconstituted from the butanol extract (0.8 mg protein) and *E. coli* phospholipids (10 μmole P) as described in the METHODS, and suspended in 1 ml of 0.2 M NaCl (pH 8.0). A portion (500 μl) of this suspension (○) was incubated for 3 min at 25°, mixed with [^{14}C]proline (final 7 μM), and incubated further for 3 min. The reaction was started by adding valinomycin (downward arrow) at a final concentration of 1 μM. At intervals, samples of 50 μl were collected on filters and washed. To another portion of the suspension (●), unlabeled L-proline (final 1.4 mM) was also added at the time indicated by an upward arrow, and uptake was measured as above.

phospholipids (40 mM of phosphorus in chloroform) was added and the mixture was evaporated as described above. In some experiments cholesterol was also added. The residue was dispersed in 1 ml of 0.1 M potassium phosphate (pH 8.0)-0.1 M KCl using a vortex mixer, and sonicated for 2 min in a bath-type sonofier (Bransonic 220). The reconstituted, K^+-loaded vesicles were precipitated by centrifugation for 40 min at 110,000 x*g* and 15°, rinsed with 1 ml of 0.2 M NaCl (adjusted to pH 8.0), gently resuspended in 1 ml of the same NaCl solution, and freed from large aggregated particles by low speed centrifugation (2,000 x*g*, 5 min). The suspension of vesicles was kept at room temperature and assayed for uptake of proline within 2 hr of preparation. Under these conditions about 50% of the protein of the butanol extract was recovered in the vesicles.

Assay of Proline Uptake --- Assays were conducted at 25°. The suspension of reconstituted, K^+-loaded vesicles (500 μl) was incubated for 3 min, and then [^{14}C]proline (10 μl) and the other compounds indicated were added. After further incubation for 3 min, 5 μl of valinomycin in ethanol (final concentration, 1 μM) was added to the mixture to start the uptake reaction. At various times, samples (50 μl) of the mixture were applied to a membrane filter (Sartorius M1336, pore size 0.45 μm), and the filter was washed with 5 ml of 0.2 M NaCl (pH 8.0). Radio-

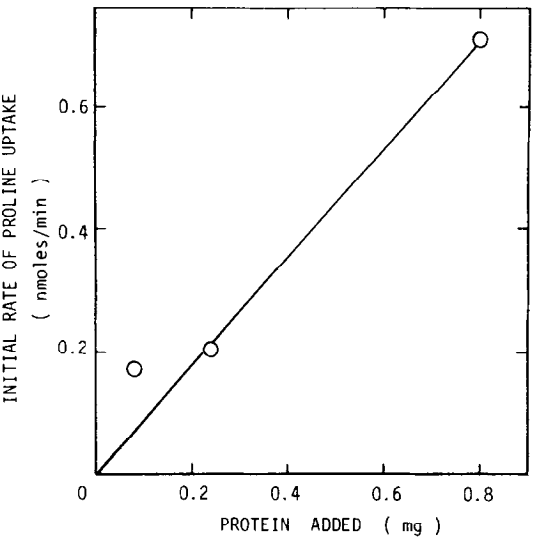

Figure 2. Initial rate of valinomycin-induced proline uptake by reconstituted, K^+-loaded vesicles as a function of the amount of protein added. The vesicles were prepared from the butanol extract containing the indicated amounts of protein and *E.coli* phospholipids (10 μmole P), as described in the METHODS. Initial rates of uptake were calculated from the values observed 7 sec after adding valinomycin. The concentration of [^{14}C]proline in the reaction mixture was 4 μM.

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activity was measured in Bray's solution (22). Observed values were corrected for nonspecific adsorption of isotope on the filter.

Sephadex LH-20 Column Chromatography --- The butanol extract (25 ml) was kept in an ice bath for 8 hr and the resulting precipitate was removed by centrifugation for 15 min at 24,000 *xg* and 0°. The supernatant solution, containing 17 mg of protein, was evaporated nearly to dryness in a rotary evaporator at about 35°. Then it was dissolved in 2 ml of *n*-butanol-glacial acetic acid (adjusted to pH 4.3 with pyridine), 30:11, and applied to a column of Sephadex LH-20 (1 x 66 cm) which had been equilibrated with the same solvent. The column was developed with the same solvent at about 20°.

Analytical Methods --- Protein was determined by the method of Lowry *et al.* (23) in the presence of 0.5% sodium dodecyl sulfate with bovine serum albumin as a standard. Lipid phosphorus was measured by the method of Gerlach and Deuticke (24)

RESULTS AND DISCUSSION

Extraction and Reconstitution --- About 2% of the protein and 30% of the phospholipid of the membranes were extracted into acidic *n*-butanol under the conditions described in the METHODS. The extract had no D-lactate oxidase activity but contained flavins and heme B as revealed by spectroscopic analyses.

Figure 1 shows the uptake of [^{14}C]proline by reconstituted, K^+-loaded ves-

Table I. Effects of various compounds on valinomycin-induced uptake of proline by reconstituted, K^+-loaded vesicles.

Compound	Concentration	Relative Activity[1]
None		100
SF6847	1 μM	0
N-Ethylmaleimide[2]	2 mM	6
p-Chloromercuribenzoate[2]	0.1 mM	45
$HgCl_2$[2]	0.1 mM	38
L-Proline	0.4 mM	9
DL-3,4-Dehydroproline	0.4 mM	38

1) Initial rates of uptake were determined as described in the text and the results are expressed as relative values. The concentration of [^{14}C]proline was 4 μM.
2) Reconstituted vesicles were treated with these reagents for 2 min at 25° before adding [^{14}C]proline.

icles prepared from the butanol extract and *E.coli* phospholipids. Rapid uptake occurred on addition of valinomycin (time 0). This uptake reached a maximum after about 7 sec, and then gradually decreased to the base line. Vesicles reconstituted using various amounts of the butanol extract and a fixed amount of *E.coli* phospholipids showed similar profiles of uptake. The initial rates of uptake were calculated from the values observed 7 sec after adding valinomycin. The rates were nearly proportional to the amount of the protein added (Fig. 2). The specific activity of uptake, calculated on the basis of the protein content of the vesicles, was 1.8 nmoles proline/min/mg protein. This value is significantly larger than that reported for valinomycin-induced uptake by K^+-loaded membrane vesicles of *E.coli* (3). Liposomes prepared without the butanol extract showed no uptake of proline on addition of valinomycin. Cholesterol, added to the *E.coli* phospholipids at a molar ratio of 1:40, had no significant effect on the uptake by the reconstituted vesicles. No protein fraction with binding activity for proline could be solubilized from *E.coli* membranes using a detergent such as deoxycholate, Brij 58, lauroylsarcosinate, or Triton X-100, and a fraction extracted with an

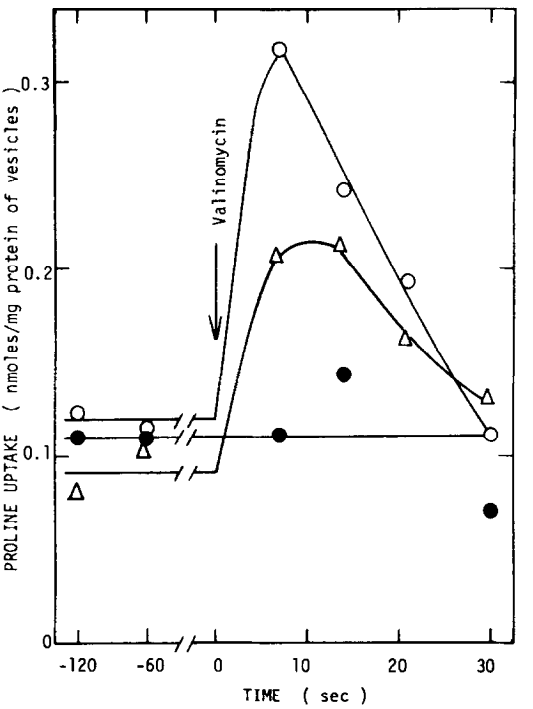

Figure 3. Proline uptake by reconstituted, K^+-loaded vesicles prepared from butanol extracts of *E. coli* JE2133 (○), and of mutants defective in proline transport, PT21 (●) and PT22 (△). Experimental procedures were as for Fig. 1 except that 4 μM [^{14}C]proline was used. The amounts of proteins from strains JE2133, PT21, and PT22 used for reconstitution were 0.98 mg, 0.81 mg, and 0.88 mg, respectively.

other organic solvent, isoamylalcohol, showed less proline uptake when reconstituted into K^+-loaded vesicles than the butanol extract.

It was found that the base line for valinomycin-induced uptake, shown in Fig. 1, reached a steady level within 1 min after adding [^{14}]proline. This fraction of the radioactivity was not affected by the presence of 160-fold excess of unlabeled L-proline but it was lost when the filter was washed with solution containing 0.08% Triton X-100 (data not shown). Thus equilibration of the internal space of the reconstituted vesicles with external [^{14}C]proline was achieved, possibly by a process of nonspecific diffusion, before uphill active uptake was initiated by valinomycin. It seems probable that traces of organic solvents remaining in the evaporated extract increased the nonspecific permeability of the re-

constituted vesicles. This increased permeability of the vesicles was probably the reason for the rapid decrease in accumulated [^{14}C]proline, shown in Fig. 1. Thus the extent of removal of organic solvents from the extract seems to affect the reconstitution of functional vesicles.

Properties of Proline Uptake --- Table I shows that the uptake of proline induced by valinomycin was completely inhibited by 1 μM of SF6847, a proton conducting uncoupler (16), indicating that it depended on the membrane potential (negative inside). It was also inhibited by incubating the vesicles with sulfhydryl reagents, such as *p*-chloromercuribenzoate, N-ethylmaleimide, and $HgCl_2$ (Table I). These results are comparable to those on proline uptake by isolated membrane vesicles (3, 25).

To examine whether the uptake induced by valinomycin was catalyzed by a transport carrier, the substrate specificity of the uptake was studied. As shown in Table I, the uptake was inhibited by adding 100-fold excess of L-proline or its analogue DL-3,4-dehydroproline simultaneously with [^{14}C]proline. Addition of L-proline after valinomycin caused rapid efflux of the accumulated [^{14}C]proline (see Fig. 1). These results are consistent with the substrate specificity of the proline transport system of intact cells and membrane vesicles (25-28).

Experiments on mutants defective in proline transport confirmed that proline uptake induced by valinomycin was catalyzed by a transport carrier: as shown in Fig. 3, reconstituted vesicles of PT21 showed no uptake of proline on addition of valinomycin, and those of PT22 showed less uptake activity than those of JE2133.

The butanol extract contained much phospholipid besides protein (7.2 mg phospholipid/mg protein). This phospholipid was nearly all removed by chromatography of concentrated butanol extract on a column of Sephadex LH-20 as described in the METHODS. The proline carrier was recovered in the fraction of the eluate containing mainly protein (0.8 mg phospholipid/mg protein; Figure not shown). The specific activity of uptake in this fraction increased about 4 times that of the original extract. The reconstituted, K^+-loaded vesicles prepared from the butanol extract also took up glutamate and cysteine, but not lysine or serine on addition

of valinomycin. Large scale purification of the proline carrier and its separatio from materials with other uptake activities are in progress.

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